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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Thrombocyte-Stabilizing Factor IX-Fragments, Their Preparation and Use and Drugs Containing These

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ABSTRACT OF THE DISCLOSURE

Fragments of the blood coagulation factor IX with intact EGF-like and Gla-domain without factor IX-coagulation activity, chemically unmodified and with a molecular weight between about 12,000 and 50,000, obtainable by proteolytical cleavage as a drug with antithrobotic efficacy and the method for their preparation, their usage for the preparation of drugs and these drugs themselves.

# Thrombocyte-stabilizing Factor IX-fragments, their preparation and use and drugs containing these.

The invention refers to fragments of the coagulation factor IX which contain intact GLA- and EGF-like domain as a substance for stabilizing platelets in vivo.

Furthermore the invention refers to the production of these substances and drugs containing these substances.

Haemostasis is a complex process which includes the participation of several plasma proteins, blood cells and vessel components. Its physiological function is to protect the organism against blood loss, while performing blood coagulation and at the same time avoiding escalation of the coagulation process. Thereby hemostasis maintains the bloodflow in the whole organism.

Disorder in haemostasis might lead to intravascular occlusions (thrombosis).

Prophylaxis and treatment of thrombosis diseases, like heart attack, venous thrombosis, DIC and stroke are very complicated, since inhibition of blood coagulation also causes risks of life-threatening bleeding complications.
Well known are three classes of antithrombotic substances. These are thrombin inhibitors, vitamin-K-antagonists and inhibitors of platelet-aggregation.

In clinical praxis, heparin catalysing the complex-formation of coagulation proteases with AT III is the most used anticoagulant. The anticoagulant action of heparin mainly consists of thrombin inhibition.

Using heparin causes risks of bleeding complications, since complete thrombin inhibition also inhibits extrinsic coagulation. Therefore heparin dosage has to be adjusted exactly by means of in vitro coagulation tests. The use of heparin is a difficult balance between an effective antithrombotic action without risking a bleeding disorder.

1-5 % of heparin treated patients show a decrease in platelet count.

The most potent natural inhibitor of thrombin is hirudin, a leech protein. Hirudin forms a stoechiometric complex with thrombin. Up to now no clinically tested antidot exists, therefore bleeding complications can not be excluded.

Further anticoagulants are the vitamin-K-antagonists, which like Warfarin prevent the gamma-carboxylation in order to decrease plasma level of active vitamin-K-depending coagulation factors II, VII, IX and X. Full anticoagulatory effect occur with a delay of 36-72 hours after injection of antagonists. Thus they are not suited for the treatment of acute thrombosis. Use of these substances might cause bleeding complications equally to those seen using heparin.

Platelet aggegation inhibitors, for example acetyl salicylic acid belong to the third class of antithrombotic substances applied

today.

They are not able to inhibit activation of plasmatic coagulation factors and their action is predominantly limited to the treatment or phrophylaxis of arterial thrombosis.

These examples show that treatment of thrombosis using available drugs has some decisive disadvantages. Mainly the not well controlled inhibition of the extrinsic coagulation which protects against blood loss and the fact that the drugs used today interfere with the coagulation cascade at its end and may result in uncontrolled bleeding complications are disadvantages of these drugs.

In general it is desirable for the antithrombotic therapy to interfere at an early stage with the coagulation system in order to prevent irreversible processes (e.g. platelet activation), because otherwise the therapeutic treatment has only a limited effect in preventing possible damage.

The aim of the invention was on the one hand to inhibit the plasmatic coagulation and at the same time prevent a decrease in platelet count, while the function of the protecting extrinsic coagulation remains intact.

According to the invention the problem was solved by the preparation of proteolytical fragments of factor IX, containing an intact gamma-carboxyglutamate-domain (Gla) and an epidermal growth facter-like domain (EGF) and having a molecular weight of about 12.000 to 50.000 Dalton.

Surprisingly it was found, that the factor IX fragments according

to the invention are able to inhibit the factor IXa induced decrease of the thromobcyte count in vivo. The factor IX has an imported role in hemostasis because the "extrinsic" and the "intrinsic" pathway are crossing here (Mann, K. G., Jenny, R. J. et al., Annu. Rev. Biochem, 57 (1988), 915-956). Factor IX is a vitamin-K-dependent plasma glycoprotein with 415 amino acids. It contains an amino terminal domain with gamma-carboxy-glutamic acid residues and two domains, containing sequences which resemble these from epidermal growth factor (EGF) (Thompson, A. R., Blood, 67, (1986), 565-572).

According to one aspect of the present invention, there is provided drug with antithrombotic efficacy on the basis of proteolytic fragments of the human blood coagulation factor IX, where the fragments a.) are containing the Gla-domain and at least one EGF-like domain, b.) are showing no factor IX coagulation activity, c.) are not chemically modified and d.) have a molecular weight of about 12,000 to 15,000 D.

According to a further aspect of the present invention, there is provided method for the preparation of a drug as defined above where the factor IX is bound on a carrier which binds the factor IX on the amino terminal end, the factor IX is cleaved proteolytically, the fragments are eluted from the carrier, purified and if necessary lyophilized.

According to another aspect of the present invention, there is provided method for the preparation of a drug as defined above where the factor IX is cleaved proteolytically in solution after the addition of protecting substances and

the fragments are separated, purified and if necessary lyophilized.

According to a still further aspect of the present invention, there is provided an antithrombotic compound comprising a proteolytic fragment of human blood coagulation factor IX wherein said fragment a.) contains a Gla-domain and at least one EGF-like domain, b.) shows no factor IX coagulation activity, c.) is not chemically modified and d.) has a molecular weight of about 12,000 to 50,000 D.

According to another aspect of the present invention, there is provided a use of a drug as defined above in the treatment of a thrombotic disease.

The results of the previous experiments about the efficacy and the inhibition of factor IX or factor IXa in difficult thrombosis models are based on the competitive inhibition of the binding to the factor IXa receptors of endothelial cells. In these described coagulation processes the thrombocyte-mediated coagulation played only a minor role.

In EP 0 263 529 it was demonstrated that peptides of the EGF-like domain of factor IX or a sequential part of it with a molecular weight of 500 to 1,000 Dalton can inhibit the generation of a thrombus in Wessler's stasis-thrombosis-model. The basis for this was the inhibition of the interaction of the injected factor IXa with endothelial cells in an isolated part of a vessel by the EGF-like sequence containing peptide.

In Wessler's stasis animal model the generation of a thrombus formed under total stasis of the blood stream in an isolated part of the vessel is the decisive characteristic. In this model the

thrombocyte-mediated coagulation of the physiological blood stream is of minor importance.
It is unknown, if the EGF-like peptides have any influence on the physiological thrombocyte-mediated coagulation with the simultanous decrease of the thrombocyte count.

To get a significant inhibition of thrombus generation very high concentrations of peptides were neccessary. The mass relation peptide to factor IXa was about 1660 to 1, which means a molecular relation of peptide to factor IXa of 65.000 to 1.

Disadvantages of the use of such small peptides are also their short half-life time of a few minutes.

Astermark and Stenflo, J, Biol. Chem., 260, 2438-2443 examined different domains of bovine factor IX, their effects on blood coagulation in vitro and their interaction with factor IX-receptors on bovine endothelial cells in vitro. An efficacy of the isolated factor IX-domains was not described. With native factor IX it was not possible to inhibit thrombosis (Benedict, Ryan, et al. J. Clin.Invest., 88, (1991), 1760-1765).
With chemically inactivated factor IXa it was possible to inhibit the formation of fibrin clots in tumor tissue of mice in which tumor necrosis factor was injected. With the zymogen factor IX no difference to the control was detectable (Tijburg, Ryan et al., J. Biol. Chem. 266, (1991), 12067-12074).

In other examinations it was shown that endothelial cells and thrombocytes in the presence of factor VIII and factor X have high

affinity binding sites for factor IXa, but not for factor IX, which are important for the further course of the coagulation (Stern, Nawroth, J. Biol. Chem. 260, 6717-6722; Hoffmann, Monroe, Thromb. and Hemost., 68, (1993), 74-79 and Ahmad, Rawala-Sheik, J. Biol. Chem., 264, (1989), 20012-20016).

Because of this knowledge about the characteristics of factor IX obtained until now, it could in no case be expected that the use of factor IX fragments according to the invention makes the stabilization of thrombocytes possible.

Surprisingly it was found that factor IX fragments according to the invention lead to a stabilization of thrombocytes, although normally a massive participation of thrombocytes takes place under physiological conditions during intravascular fibrin formation which leads to a consumption of thrombocytes.

Preparation of factor IX fragments

For the preparation of the factor IX fragments according to the invention purified factor IX-fractions from plasma as well as other, e.g. by genetic engeneering prepared factor IX containing solutions can be used as source material.
According to the invention the factor IX is treated with proteases under conditions where the Gla- and at least one EGF-like domain stay largely intact and where fragments of factor IX are formed with a molecular weight of about 12.000 to 50.000 D. This can be achieved e.g. with proteases having the same or a similar specificity like e.g. Russel's viper venom and where the factor IX

fragments should have no residual factor IX activity and shouldn't be chemically modified.

If proteases with a broad specificity are used like e.g. chymotrypsin or trypsin the proteolysis is performed under conditions where the amino terminal end of factor IX is protected. Preferrably the factor IX is bound reversible, that means non covalent, with its amino terminal end onto a solid support and treated in the immobilized form with the proteases. After that the antithrombotic efficaciuos fragments of factor IX are eluted from the support.

As supports, anion exchangers or affinity supports with affinity to factor IX on its amino terminal end, can be used. Especially preferable the factor IX is bound onto polymers carrying alpha-hydroxy-amino-propyl-groups, e.g. Fractogel Amino-TSK.

Another possibility for the preparation of the factor IX fragments according to the invention is the proteolytical cleavage of factor IX in solution with proteases like e.g. chymotrypsin or trypsin, preferably immobilized on a carrier, where the amino terminal end of factor IX is protected with low molecular substances binding to the Gla- and EGF-like domain. Preferably low molecular amines and especially preferable 1-amino-2-propanol are used as such protecting substances. Also possible is the use of other protecting substances like e.g. protamine.

The proteolytic factor IX fragments may be treated with mild reducing agents, e.g. dithiothreitol under gentle conditions. Thus it is principally possible to isolate inhibitory active factor IX

fragments which are not covalently modified and have no factor IX activity.

It is known, that with the conventional treatment of the factor IX with alpha-chymotrypsin, fragments are generated which have no intact Gla- or EGF-like domains or where the yield of fragments is only very poor (Wildgoose et al., Biochem and Biophys. Res. Comm., 152 (1988), 1207-1212). Controlled proteolytic cleavage of factor IX was a problem because a mixture of different fragments was always generated.

Using the method according to the invention it was for the first time possible to prepare efficacious factor IX fragments in a technically reproducible manner and with high yield in a pure form.

The advantage of the preparation methods according to the invention are among others:

1. The avoidance of highly toxic protease inhibitors (e.g. diisopropyl-
fluorophosphate) for the inactivation of the proteases used for the cleavage.

2. The avoidance of a chemical modification of the factor IX fragments.

3. A gentle proteolytical cleavage of the factor IX because of the fixation
of the amino terminal end onto a matrix, preferrable a matrix

containing 2-hydroxy-amino-propyl groups.

4. A gentle proteolytical cleavage of the factor IX because of the use of low molecular amines for protection of the amino terminal domains.

5. A chromatographic separation of the antithrombotic efficacious factor IX fragments from ineffective fragments.

Example 1:

Preparation of factor IX-fragments from native factor IX, which is bound during the proteolytic cleavage with alpha-chymotrypsin onto Fractogel Amino TSK.

A 50 ml column with Fractogel Amino-TSK (Merck, Darmstadt), a copolymerisate of glycidylmethacrylat, pentaerythroldimethacrylate and polyvinylalcohol with alpha-hydroxyl-amino-propyl-groups was equilibrated with a tris-HCL-buffer (0,05 M Tris-HCl, 0,05 M NaCl, pH 7,3). 16 mg of factor IX with a specific activity of 110 U/mg dissolved in Tris-HCl-buffer were applied onto the column. Subsequently the column was washed with the same buffer to which 10 mM calcium chloride was added.

For the fragmentation the bound Factor IX was incubated with chymotrypsin, dissolved in the calcium-containing tris-HCl-buffer. The concentration of the protease in the buffer was 0,025 mg/ml.

The column was washed with the same buffer with a flow rate of 100 ml/h. After one column-volume eluate, a protein fraction was eluted containing chymotrypsin and factor IX fragments which have no affinity to the Fractogel Amino-TSK-column under the chosen conditions. The fraction containing protease was rejected.

The column is washed with 2.5 column-volumes of the tris-HCl-chymotrypsin-buffer without protease and calcium until no protein was eluted from it.
In a further step the matrix was washed with tris-HCl-buffer (0.05 M tris-HCL, 0.15 M NaCl, pH 7.3) until no protein was eluted.
For the elution of the efficacious factor IX fragments the NaCl concentration of the tris-HCl-buffer was increased to 0.5 M. The desired fraction was eluted with a volume of 70 ml. The factor IX fragments were dialyzed against 75 mM NaCl and subsequently lyophilized.
The fragments had no more factor IX activity and inhibited the factor X activation in the presence of endothelial cells as well as the coagulation of factor IXa containing plasma under participation of phospholipids in vitro. On the other hand they didn't inhibit the coagulation of a plasma sample to which thrombin was added.

In SDS-gel-electrophoresis of the fragment-fraction under non-reducing conditions two bands with a molecular weight of 47.000 and 34.000 Dalton were detectable, which could be assigned to the amino terminal end of factor IX.
The yield of efficacious factor IX fragments in relation to the applied factor IX protein was 60 %.

Example 2:

Preparation of factor IX-fragments from native factor IX, which is bound during the proteolytic cleavage with trypsin on Fractogel amino TSK.

10 mg factor IX were treated in the same way as in example 1, with the difference that trypsin instead of chymotrypsin was used in a concentration of 0.05 mg/ml.

In SDS-gel electrophoresis under reducing conditions a main band of approximately 33.000 Dalton was detectable which could be assigned to the amino terminal end of factor IX.

The yield of efficacious factor IX fragments in relation to the applied factor IX protein was 57 %.

The factor IX fragments had no more factor IX activity. In vitro they inhibited the factor X activation in the presence of endothelial cells as well as the coagulation of factor IXa containing plasma under participation of phospholipids.
The coagulation of a plasma sample to which thrombin was added was not inhibited.

Example 3:

Preparation of factor IX-fragments in the presence of 1-amino-2-propanol

To 16 mg factor IX with a specific activity of 110 U/mg dissolved in 60 ml tris-HCl-buffer (0,05 M Tris-HCl, 0,15 M NaCl, pH 7,5) 10 mM 1-amino-2-propanol were added. This solution was applied to a 15 ml column with alpha-chymotrypsin-Sepharose with a flow rate of 200 ml/h. Every 15 minutes the remaining factor IX activity in the solution was determined with the one stage assay. When no remaining factor IX activity was detectable in the solution the column was washed with buffer until no more protein was eluted from the column.
The solution with the factor IX fragments was dialyzed against tris-HCl (0,05 M Tris-HCl, 0,2 M NaCl, pH 7).

A purification of the desired factor IX fragments was achieved by chromatography with a carrier with 2-hydroxyl-amino groups (Fractogel Amino-TSK). A 50 ml column with Fractogel Amino-TSK was equilibrated with tris-HCl-buffer (0,05 M Tris-HCl, 0,1 M NaCl, pH 7). The solution with the factor IX fragments was diluted with two parts of aqua dest and applied to the column with a flow rate of 300 ml/h. The antithrombotic effective factor IX fragments were bound onto the column whereas the uneffective fragments and other impurities were eluted.

After a washing step with tris-HCl-buffer (0,05 M Tris-HCl, 0,15 M NaCl, pH 7) the antithrombotic effective fragments were eluted with tris-HCl-buffer (0,05 M Tris-HCl, 0,5 M NaCl, pH 7) in a volume of 40 - 60 ml. They have the same characteristics as in

example 1. Their molecular weight was around 50.000 Dalton which could be assigned to the amino terminal end of factor IX.

After a repeated dialysis against 0,05 M Tris-HCl, 0,075 M NaCl, pH 7 the factor IX fragments were lyophilized.

Example 4:

Preparation of factor IX-fragments from activated factor IX, which was bound to Fractogel Amino-TSK during the proteolysis.

Russel viper venom (RVV, Sigma, Heidelberg) was immobilized on CNBr-activated Sepharose 4 B according to the instruction of the manufacturer (Pharmacia, Sweden). 1 ml gel contained 1.5 mg RVV. A factor IX solution with a specific activity of 110 U/mg was applied onto the column until no further increase of activity was detectable. The solution was dialyzed against tris-HCl-buffer (0.05 M tris-HCl, 0.05 M NaCl, pH 7,3).

A 30 ml column with Fractogel Amino-TSK was equilibrated with tris-HCl-buffer. The activated factor IX-solution was applied onto the column and the column was washed with the same buffer to which 10 mM calcium chloride was added.
To the buffer with calcium chloride, alpha-chymotrypsin was added in a concentration of 0.015 mg/ml and this solution was pumped in circulation over the column with a flow rate of 75 ml/h. After one column volume eluate, a fraction was obtained containing alpha-chymotrypsin and fragments of factor IXa, without affinity to

Amino-TSK.

After washing with 3 column volumes of the chymotrypsin-containing buffer the column was washed with tris-HCl-buffer without protease until no more protein was eluted.

The matrix was washed with 0.05 M tris-HCl, 0.15 M NaCl, pH 7 until no more protein was eluted and subsequently eluted with 0.05 M tris-HCl, 0.5 M NaCl, pH 7.

The factor IX fragments were dialyzed against 0.075 M NaCl and lyophilized.

The yield of efficacious factor IX fragments in relation to the applied factor IX protein was 60 %.

In SDS-gel electrophoresis under non-reducing conditions one main band with a molecular weight of 31.000 Dalton was detectable, which could be assigned to the amino terminal end of factor IX.

The factor IX fragments had no more factor IX activity. In vitro they inhibited the factor X activation in the presence of endothelial cells as well as the coagulation of factor IXa containing plasma under participation of phospholipids.

On the other hand they didn't inhibit the coagulation of a plasma sample to which thrombin was added.

Surprisingly an inhibition of the factor IXa induced drastic decrease of thrombocytes in vivo could be achieved by the factor IX fragments according to the invention. Factor IXa is a strong thrombogen which induces intravascular thrombosis in rabbits.

Animal experiments with rabbits

To 5 anesthetised rabbits 0.12 mg/kg body weight FIXa was injected with a cannula in the ear vein.

To another 5 animals a dose of 1.8 mg/kg body weight factor IX fragments from example 1 were injected 5 to 10 minutes before the application of factor IXa.

5 control animals recieved 0.2 ml/kg body weight 0.9 % NaCl-solution instead of Factor IXa.
During the whole period of the experiment of 60 minutes the arterial blood pressure, the breething frequency and the standard EKG were registered and in fixed intervalls arterial blood was drawn in citrate tubes. In the drawn blood the thrombocyte count and in the plasma of some animals the APTT and thrombin time was determined.

Except the preparation of the rabbits for the experiment the vessel system of the animals wasn't impaired. The physiological blood flow in the vessels was ensured.

In the control group a slight decrease of the thrombocyte count was detectable. This is an indication that even the preparation of the animals and the narcosis has an influence on the stability of the thrombocytes.

In the animals recieving the factor IXa compared to the NaCl-control group a drastic, significant drop of the thrombocyte count was detectable. The animals showd symptoms of a disseminated intravasale coagulation (DIC). A heavy decrease of blood pressure

as well as strong deviations in the breething frequency and EKG occured.

A distinct indication for an activation of the coagulation was the coagulation of some of the blood samples in the citrate tubes and the shortening of the APTT respectively the increase of the thrombin time, which indicated a consumption of fibrinogen and the formation of fibrin clots in the vessels.

The rabbits which recieved the factor IX fragments according to the invention 5 - 10 minutes before the factor IXa dose showed none of the above described incompatibility reactions. The thrombocyte count remained surprisingly in the range of the control group. In no case a coagulation in the test tubes was observed. The APTT and the thrombin time remained relatively constant. This means that the coagulation activation was prevented, without affecting the protecting extravascular coagulation.

The single dose of 2 mg/kg body weight factor IX fragments showed no signs of incompatibility reactions and kept the thrombocyte count above the level of the control animals. The consequences of the preparation of the animals and the narcosis on the thrombocyte count were avoided by the factor IX fragments.

With the factor IX fragments according to the invention it is possible to avoid a massive thrombocyte-mediated coagulation activation in vivo and to stabilize at the same time the thrombocytes. The protecting extravascular coagulation is not affected by this treatment.

Claims

1. Drug with antithrombotic efficacy on the basis of proteolytic fragments of the human blood coagulation factor IX, where the fragments

a.) are containing the Gla-domain and at least one EGF-like domain,

b.) are showing no factor IX coagulation activity,

c.) are not chemically modified and

d.) have a molecular weight of about 12,000 to 50,000 D.

2. Drug for the prophylaxis and therapy of thrombotic diseases, containing or consisting of the preparation according to claim 1.

3. Method for the preparation of a drug according to claim 1, where the factor IX is bound on a carrier which binds the factor IX on the amino terminal end, the factor IX is cleaved proteolytically, the fragments are eluted from the carrier, purified and if necessary lyophilized.

4. Method according to claim 3, where the solid carrier is an anion exchanger or an affinity support with affinity to the amino terminal end of factor IX.

5. Method according to claim 3 to 4, where the solid support is a carrier with alpha-hydroxy-amino-propyl-groups as functional groups.

6. Method according to claim 3 to 4 where the solid support is a copolymerisate of glycidylmethacrylat, pentaerythrol dimethacrylate and polyvinylalcohol with alpha-hydroxy-amino-propyl-groups.

7. Method for the preparation of a drug according to claim 1, where the factor IX is cleaved proteolytically in solution after the addition of protecting substances and the fragments are separated, purified and if necessary lyophilized.

8. Method according to claim 7, where the protecting substances are low molecular amines.

9. Method according to claim 7 and 8, where the factor IX is cleaved proteolytically after the addition of 1-amino-2-propanol.

10. Method according to claim 7 to 9, where the protease is immobilized on a solid support.

11. Method according to claim 3 to 10, where the factor IX is activated to factor IXa before the proteolytic cleavage.

12. Method according to claim 3 to 11, where the protease is alpha-chymotrypsin.

13. Method according to claim 3 to 11, where the protease is trypsin.

14. Method according to claim 3 to 13, where the source material is factor IX from human plasma or factor IX-containing plasma fractions or from cell culture media.

15. Use of the preparation according to claim 1 to prepare a drug for the prophylasix or therapy of thrombotic diseases.

16. An antithrombotic compound comprising a proteolytic fragment of human blood coagulation factor IX wherein said fragment.

a.) contains a Gla-domain and at least one EGF-like domain,

b.) shows no factor IX coagulation activity.

c.) is not chemically modified and

d.) has a molecular weight of about 12,000 to 50,000 D.

17. A use of a drug according to claim 1 in the treatment of a thrombotic disease.

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